

Repair of Traumatic Skeletal Muscle Injury with Bone-Marrow-Derived Mesenchymal Stem Cells Seeded on Extracellular Matrix

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Skeletal muscle injury resulting in tissue loss poses unique challenges for surgical repair. Despite the regenerative potential of skeletal muscle, if a significant amount of tissue is lost, skeletal myofibers will not grow to fill the injured area completely. Prior work in our lab has shown the potential to fill the void with an extracellular matrix (ECM) scaffold, resulting in restoration of morphology, but not functional recovery. To improve the functional outcome of the injured muscle, a muscle-derived ECM was implanted into a $1 \times 1 \text{ cm}^2$, full-thickness defect in the lateral gastrocnemius (LGAS) of Lewis rats. Seven days later, bone-marrow-derived mesenchymal stem cells (MSCs) were injected directly into the implanted ECM. Partial functional recovery occurred over the course of 42 days when the LGAS was repaired with an MSC-seeded ECM producing $85.4 \pm 3.6\%$ of the contralateral LGAS. This was significantly higher than earlier recovery time points ($p < 0.05$). The specific tension returned to $94 \pm 9\%$ of the contralateral limb. The implanted MSC-seeded ECM had more blood vessels and regenerating skeletal myofibers than the ECM without cells ($p < 0.05$). The data suggest that the repair of a skeletal muscle defect injury by the implantation of a muscle-derived ECM seeded with MSCs can improve functional recovery after 42 days.

Introduction

TRAUMATIC INJURY TO A SKELETAL MUSCLE that involves the loss of a volume of the tissue presents a unique challenge to the normally robust regenerative capacity of skeletal muscle. Injuries involving volumetric muscle loss (VML) are often seen in military personnel wounded in action by gunshots and blasts.^{1,2} In response to damage, skeletal muscle goes through a well-defined series of events including inflammation, repair, and remodeling (for review^{3,4}). Ultimately, repair is the result of resident muscle stem cells, known as satellite cells, which proliferate, differentiate, and fuse with existing myofibers or form new myofibers.^{5,6} The normal repair mechanisms, however, are not sufficient for the repair of VML.^{7,8} The remaining myofibers are incapable of bridging across gaps created by the injury, and scar tissue will fill the area or the muscle will remodel such that an area is permanently devoid of tissue.⁹ In the case of injuries such as these, the victim is often left with a permanent functional and morphological handicap.

Complete repair of VML is dependent on the ability of an implant to fill the void in the tissue while allowing for the

growth and development of functional myofibers, blood vessels, and nerves. The current standard of care for these injuries is to transfer autologous tissue (muscle flaps) using donor tissue from other areas of the victim's body. Recent reports describe functional free muscle transplantation in the forearm¹⁰ and elbow,¹¹ but these procedures are associated with significant donor-site morbidity and are not yet applicable to large defects of load-bearing muscles. The implantation of a scaffold seeded with progenitor cells to repair the defect and allow for the growth of new tissue into the area could be a way around the morbidity associated with autologous tissue transfer.

The extracellular matrix (ECM) is a scaffold comprised predominantly of collagen, which is critical in the development and growth of skeletal muscle.¹² Skeletal muscle tissue can be decellularized such that all that remains is a three-dimensional ECM.^{13,14} Using a three-dimensional ECM derived from skeletal muscle as a scaffold is advantageous because differentiation of muscle progenitor cells is stimulated by numerous factors, including their three-dimensional configuration, and chemical and mechanical environment.^{15,16} The decellularized ECM serves as a platform for

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the growth of functional muscle, blood vessels, and nervous tissue.^{13,17–19} The three-dimensional configuration of the ECM allows it to translate linear forces throughout the construct, thus applying tension through the adhesion molecules to developing cells and further simulating the developmental environment of skeletal muscle.

In myocardial damage models, defects repaired with ECM-derived implants incorporate myocardial cells and improve function.^{20,21} Implanted into skeletal muscle *in vivo*, the ECM is capable of supporting limited growth of new myofibers while maintaining the overall morphology of the area, but functional recovery does not occur.^{8,22,23} The addition of muscle precursor cells to acellular ECM implants used to repair abdominal wall defects, however, increases the amount of muscle tissue incorporated into the ECM, although the functional significance of this has yet to be determined.^{23–27}

The full repair of a large defect in a skeletal muscle will require the growth of myofibers, but it also requires blood vessel integration and nerve innervation of the myofibers. The implantation of myoblasts or other myogenically committed cells might not be capable of regenerating vessels and nerve. A pool of cells, known as mesenchymal stem cells (MSCs), that is multipotent and easily expandable in culture is found in the red bone marrow. Bone marrow-derived cells, isolated by adherence to the plastic cell culture vials through repeated passage, are termed MSCs. These cells were originally described by Friedenstein²⁸ and are capable of differentiating into a number of other tissues, including nerve, muscle, and vascular tissue, that are necessary for viable muscular regeneration after muscle defect injury.^{29–32} Cells from the bone marrow are known to participate in skeletal muscle regeneration naturally.^{33,34} Dystrophin-positive myofibers are found in dystrophic skeletal muscle of mice after the addition of donor bone marrow cells.³⁵ Addition of bone marrow-derived MSCs aids in the functional regeneration of skeletal muscle after both crush and laceration injury.^{36,37} The addition of MSCs to acellular ECM implants in the defected myocardium has shown the ability of MSCs to differentiate into cardiomyocytes and engraft into the ventricular wall, preserving its structure and demonstrating the potential of this technique to be beneficial for cardiac and skeletal muscle regeneration.^{38,39}

Although bone marrow-derived cells can aid in the repair of injured muscle and that muscle tissue can incorporate into an implanted ECM *in vivo*, the VML models studied to date have not functionally assessed regenerating muscles to determine the physiological significance of large VML repair, especially in muscles that are load bearing and experience a significant amount of mechanical tension during regeneration. Therefore, the aim of this study was to determine the functional and morphological regeneration potential of an injured skeletal muscle with VML and subsequent surgical replacement of the lost tissue with decellularized ECM with or without the addition of bone marrow-derived MSCs.

Methods

Subjects

Male Lewis rats from colonies maintained by the Charles River Company were used in experimental procedures. The rats were approximately 6–9 months old at the beginning of

treatment and weighed at least 400 g. Rats were allowed *ad libitum* access to food (Rodent Chow; Harlan Teklad) and water. Rats were randomly assigned to experimental groups. All experimental procedures were conducted in accordance with guidelines set by the University of Texas at Austin Institutional Animal Care and Use Committee.

For all surgical procedures, rats were under general anesthesia. Animals were anesthetized with an intraperitoneal injection of sodium pentobarbital (55–65 mg/kg body weight). After all experimental procedures anesthetized animals were euthanized with an overdose bolus injection of sodium pentobarbital (80 mg/kg body weight) to the heart.

Experimental groups

Lewis rats were randomly assigned to two groups: ECM-ONLY ($n = 27$) and ECM-CELL ($n = 20$). ECM-ONLY rats were divided into four recovery groups: 7 day ($n = 6$), 14 day ($n = 6$), 28 day ($n = 6$), and 42 day ($n = 9$). Force measures for rats in the ECM-ONLY group were previously reported by Merritt.¹⁹ Rats in the ECM-CELL group were divided into three recovery groups: 14 day ($n = 6$), 28 day ($n = 6$), and 42 day ($n = 8$). Since the cells were not injected into the ECM-CELL group until 7 days postdefect/ECM implant, no 7-day recovery group was necessary. Rats in each group underwent procedures as described below.

ECM isolation

Gastrocnemius muscles were removed from donor male Lewis rats and decellularized as previously reported.¹⁹ Briefly, under sterile conditions, muscles were dissected free and placed in 4°C dH₂O water for 1 day. The muscle was placed in chloroform and continuously agitated for 4–5 days depending on size. The muscle was rinsed with water and submerged in 2% sodium dodecyl sulfate (SDS; Sigma-Aldrich) and agitated continuously. The SDS solution was changed twice per week until the cellular components were washed out. The remaining ECM was rinsed in deionized water (10:1 v/w) over several days with solution changes each day. The ECMs were then rinsed for 4 h in a 0.1 M Tris buffer solution of pH 9.0. Finally, the ECM was submerged in phosphate buffered saline with 1% penicillin/streptomycin (Sigma-Aldrich), exposed to ultraviolet light for at least 12 h, and stored at 4°C until ready for use. As previously reported, no nuclei or cytoplasm were evident within the decellularized ECMs used for implant, and the decellularization protocol removed soluble proteins and residual SDS.¹⁹

Defect creation and ECM implantation

The defect of the lateral gastrocnemius (LGAS) was created as previously described.¹⁹ Briefly, rats were anesthetized and a 2 cm incision was made on the lateral side of the lower limb parallel to the tibia. The LGAS was exposed along a 1 cm portion superior to the Achilles tendon. To create the defect, two #9 scalpel blades separated with a spacer were inserted distal to the neuromuscular junction with the proximal most scalpel blade in line with the tibial tuberosity. The LGAS was cut such that there were two lacerations through the full thickness of the muscle. The medial edge still connected to the rest of the muscle was excised with surgical

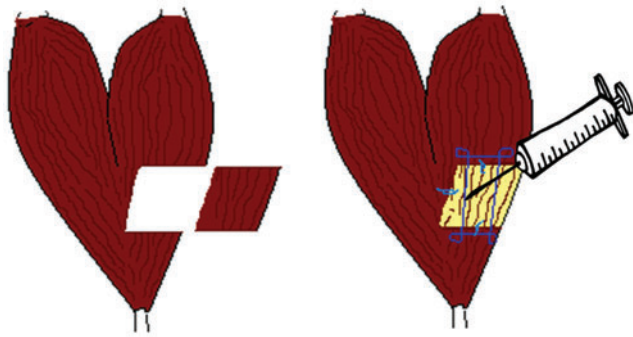


FIG. 1. Defect creation. Removal of defect from lateral gastrocnemius (LGAS). Repair with extracellular matrix (ECM) and mesenchymal stem cell injection. Color images available online at www.liebertonline.com/ten.

scissors (Fig. 1). The portion of muscle excised was weighed and measured. A portion of ECM cut to the dimensions of the defected area was implanted in the muscle using a modified Kessler stitch (5-0 Prolene; Ethicon) with simple interrupted sutures on each of the three borders to hold the cut ends together and serve as markers for later analysis. The modified Kessler stitch was used because it has been shown to be the most effective way to suture the transected muscle segments back together.⁴⁰ The wound was closed with simple interrupted polypropylene sutures (5-0, Prolene; Ethicon). The skin incision was closed with simple interrupted stitches of silk suture (4-0; Ethicon).

Isolation of bone marrow-derived MSCs and culturing

MSCs were isolated from Lewis rats using a procedure similar to that described by Friedenstein to isolate the adherent fraction of cells.²⁸ The femurs and tibias of both legs of 2 3-month-old Lewis rats were removed and trimmed of all muscle and connective tissue. The epiphyses were cut and the marrow flushed out with a Dulbecco's modified Eagle's medium (Invitrogen), 10% fetal bovine serum (Invitrogen), and 1% antibiotic/antimycotic (Invitrogen) solution. The resulting cell suspension was centrifuged, and the cells in the pellet were plated at a density of 5×10^7 cells/100 mm² on a culture dish and incubated at 37°C with 5% CO₂. The medium was changed every 2 3 days until cells reach 70% confluency. Cells were removed from the flask with 0.25% trypsin in 1 mM ethylenediaminetetraacetic acid at 37°C for 5 min, centrifuged at 1000 g, resuspended in a serum-supplemented medium, and replated at 5×10^5 cells/100 mm² on a culture dish. Culturing of the cells continued for 3 5 more passages at which time they were again removed from the flask and prepared for injection into the ECM at the defect site.

Flow cytometry was performed on cells from the fifth passage to determine the cell population. Cells in culture were washed with phosphate-buffered saline (PBS), trypsinized, and resuspended at 0.5×10^6 cells/mL in PBS with 1% bovine serum albumin. Cells were incubated for 30 min at 4°C in the following fluorochrome-conjugated antibodies: CD34-PE (Santa Cruz Biotechnologies), CD45-FITC (BD Biosciences), CD90-PerCP (BD Biosciences), and CD146-APC (R&D Systems). Cells were washed in PBS and fixed in 1% para-formaldehyde. Detection of fluorochrome labeling was

performed on a fluorescence activated cell sorting [FACS Calibur flow cytometer (BD Biosciences)]. Analysis was conducted using CellQuest Pro software at the Institute for Cellular and Molecular Biology (ICMB) Flow Cytometry Core Facility of The University of Texas at Austin.

Injection of bone marrow-derived MSCs into ECM

One week after the defect repair with ECM, rats in the ECM-CELL treatment groups were given an injection of 1.5 2 million MSCs. Cells were trypsinized and removed from the cell-culture flask, centrifuged at 1000 g, and resuspended in 300 μ L of phosphate buffered saline. The rat was anesthetized and prepared for the injection of cells. The original skin incision was opened up to observe the ECM in the defect of the LGAS. Using a 21-gauge needle, MSCs were injected in 4 6 locations throughout the ECM/defect area. After injection, the skin was once again stitched closed as described previously.

Force measurements

After the designated recovery time, the LGAS muscles were isolated and subjected to functional measurements as previously described.¹⁹ Briefly, the LGAS was isolated and the Achilles tendon with an attached portion of the calcaneus was cut and tied to the lever arm of a dual-mode servomotor (model 310 B, Aurora Scientific, Aurora, ON, Canada). The muscle was stimulated to contract utilizing a stimulator (Model 2100; A-M Systems) with leads applied to the LGAS branch of the tibial nerve 1 cm proximal to its insertion into the GAS. The muscle was kept wet in mineral oil, and the temperature maintained at 36°C with a radiant heat lamp and monitored on the muscle surface with a thermometer. The muscle length was adjusted to the length that produced the highest twitch force, and maximal twitch tension determined. The muscle was stimulated at 150 Hz and 20 V for peak tetanic tension (P_0). Each contraction was followed by 2 min of rest. The servomotor was interfaced with the computer and equipped with an A/D board (National Instruments). The data were stored and analyzed using Lab View software. After completion of contractile measurements, the muscle length was determined and it was then dissected free and weighed.

Histology and immunohistochemistry

The implant region of the LGAS muscles was removed and divided into thirds such that there was an equal sized top, middle, and bottom region for each muscle. The samples were placed in 10% neutral buffered formalin (Protocol; Fisher Scientific) for 24 h, and stored in 70% ethanol until further analysis. Samples were embedded in a Tissue Tek paraffin-embedding system before sectioning on a Reichert Jung microtome. Eighteen 5- μ m sections from each of the top, middle, and bottom regions of the defect area, for a total of 54 sections per muscle, were subjected to histologic or immunohistochemical staining. Three sections per region were stained per method, resulting in a total of nine stained sections per technique per muscle. These sections were quantified as described below and the results were expressed as mean \pm standard error for each region within each subgroup. Hematoxylin and eosin staining was performed, as

was Masson's trichrome (Sigma-Aldrich) staining to identify regions of collagen-containing ECM, as well as cells within the ECM. To observe blood vessels, the rabbit anti-human von Willebrand factor (vWF) polyclonal antibody (1:300; Kit; Dako) was used to identify endothelial cells. The signal was enhanced with biotinylated polyclonal goat-anti-rabbit IgG with streptavidin-horseradish peroxidase (HRP). Color was developed after incubation with 3,3-diaminobenzidine. Muscular infiltration into the ECM was further confirmed by immunofluorescent staining for the muscle-specific cytoskeleton protein, desmin. Sections were exposed to mouse monoclonal antidesmin antibody (1:500; Sigma-Aldrich). Sections were then incubated with F(ab')₂ goat anti-mouse IgG Fluorescein (1:100, $\lambda = 495$ nm; Thermoscientific) and counterstained with Hoescht 33258 ($\lambda = 395$ nm; AnaSpec) to identify nuclei. To identify newly regenerated myofibers, an immunofluorescent stain for the skeletal muscle transcription factor, myogenin was performed. Sections were exposed to rabbit polyclonal antimyogenin antibody (1:500; Santa Cruz Biotechnologies). Sections were then incubated with F(ab')₂ goat anti-rabbit IgG Fluorescein and counterstained with Hoescht 33258 ($\lambda = 395$ nm; AnaSpec) to determine nuclear colocalization. H&E, Masson's trichrome, and vWF sections were observed with a Nikon Diaphot microscope mounted with an Optronix Microfire digital camera interfaced with a Dell 8250 computer for storage and analysis of images. The area of each region of the ECM implant stained blue for collagen relative to red staining cytoplasm was quantified using LabView. The number of vWF-positive structures within each region of the ECM implant of each rat was counted to determine the number of blood vessels/mm². A vessel was only counted if its lumen was >20 μ m in diameter. Immunofluorescent desmin and myogenin were observed with a fluorescence microscope (Leica DM LB2) and photographed with a digital camera (Leica DFC340FX). The percent area of each region of the ECM implant positive for desmin was quantified using a LabView program developed

by Derrell Sloan (Metrosol). Additionally, the number of desmin-positive fibers was quantified on three sections within each region of the ECM in at least three animals per group at 28 and 42 days of recovery. Fibers showing nuclear localization of myogenin were counted in randomly selected fields from three sections within each region of the ECM from at least three animals per group at 28 and 42 days of recovery. Counts were performed by investigators blinded to the treatment.

Statistical analysis

Means of all measurements were analyzed utilizing unpaired Student's *t*-test and two-way ANOVA with Tukey's *post hoc* test where applicable. Data are represented as mean \pm standard error of the mean unless otherwise stated. Significance is defined as $p < 0.05$.

Results

Bone marrow MSCs were analyzed for cell surface markers by FACS analysis. Cells cultured under identical conditions and from the same passage as those injected into the ECM were consistent with described MSCs. Over 99% of cells were positive for CD90 and negative for CD45, CD34, and CD146.

The portion of the LGAS removed to create the defect was 223 ± 5 and 228 ± 6 mg wet weight for ECM-ONLY and ECM-CELL, respectively, which was nearly 20% of the mass of the LGAS. No significant differences in defect size existed between groups or within groups at the time of defect creation. Over the course of 42 days after defect creation, the overall morphology of the ECM repaired LGAS was well maintained (Fig. 2) in both groups, and no difference existed in the LGAS mass.

The maximal isometric tetanic force produced by the LGAS of the ECM-CELL group was significantly higher after 42 days of recovery than after 14 or 28 days of recovery

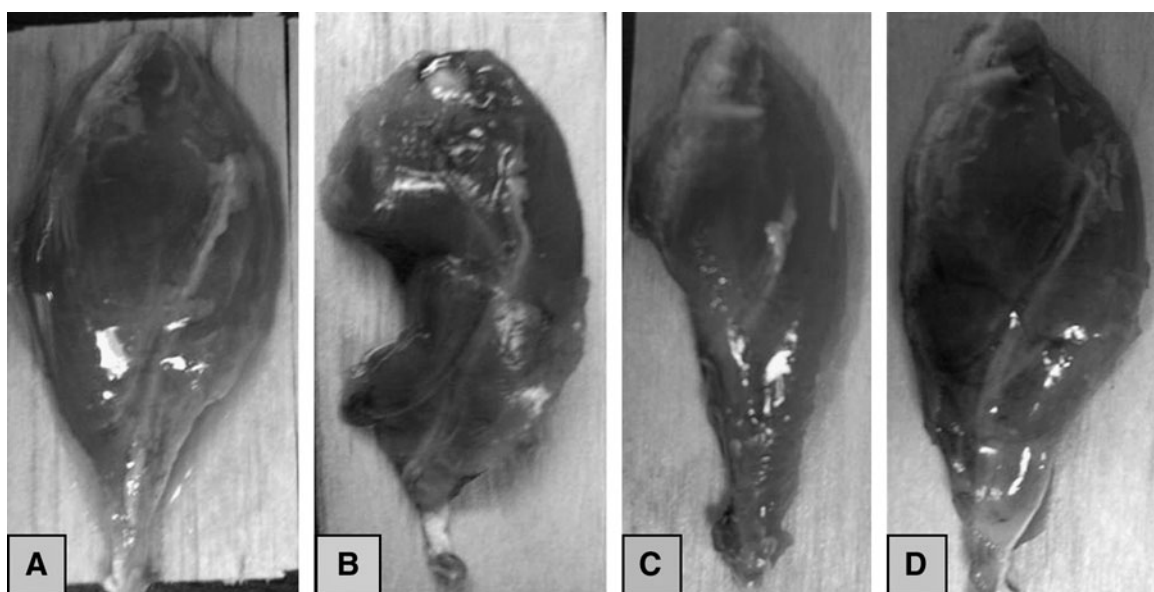


FIG. 2. Morphology. (A) Nonoperated LGAS. (B) Defect LGAS 0-day recovery. (C) Defect LGAS 42-day recovery. (D) ECM LGAS 42-day recovery.

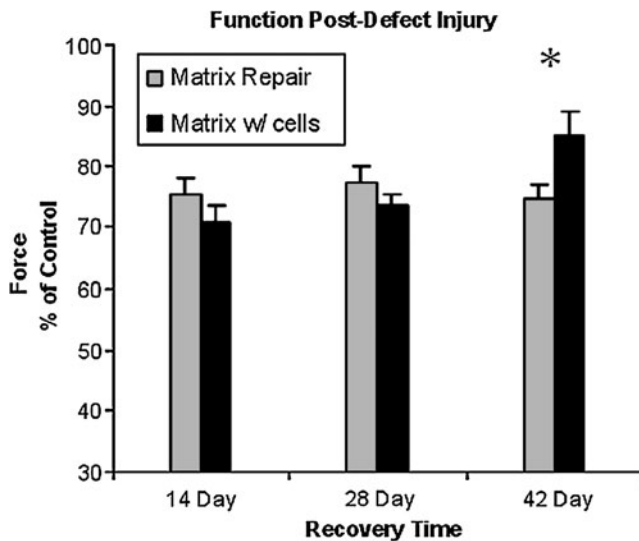


FIG. 3. Function postdefect injury. Maximal isometric, tetanic tension of ECM-repaired LGAS with or without mesenchymal stem cells relative to contralateral limb 14, 28, and 42 days postinjury. *Statistically different from all others ($p < 0.05$). Note: Includes data originally published by Merritt.¹⁹ W/, with.

($p < 0.05$) (Fig. 3). Previous work by Merritt has proven that no functional recovery of the LGAS occurs over the course of 42 days in ECM repaired LGAS defects.¹⁹ Specific tension, the maximal tetanic force per unit of cross-sectional area, of the LGAS in the ECM-CELL group increased significantly relative to the contralateral from $77\% \pm 12\%$ at 14 days of recovery to $94\% \pm 9\%$ at 42 days of recovery ($p < 0.01$).

Histological analysis of the defect area in ECM-ONLY and ECM-CELL at 14, 28, and 42 days with Masson's Trichrome stain showed increasing cellularity (Fig. 4) and the appearance of blood-vessel-like structures, which were confirmed by staining with vWF, within the ECM (Fig. 4). Quantification of the Masson's trichrome staining indicated that the cytosolic area stained red relative to blue-stained collagen area averaged across the top, middle, and bottom regions increased from recovery day 28 to recovery day 42 in both groups (Fig. 5). Values for ECM-CELL were higher than

ECM-ONLY after 42 days of recovery ($p < 0.05$). Quantification of vWF-stained blood vessel walls demonstrated an increase in the number of blood vessels within the ECM implant in the ECM-CELL compared to ECM-ONLY after 42 days of recovery ($p < 0.05$) (Fig. 5).

The muscle-specific protein desmin and the transcription factor myogenin were used to identify myofibers within the ECM implant (Fig. 6). After 42 days of recovery, regions of the ECM nearest the transected myofibers in the ECM-CELL group were densely populated with desmin-positive myofibers. The number of desmin-positive fibers per square millimeter was significantly higher after 42 days of recovery in the ECM-CELL group compared to all other groups ($p < 0.05$) (Fig. 7). Significantly more myogenin-positive nuclei were found in the MSC-seeded ECMs at 28 and 42 days of recovery (Fig. 7).

When the defect implant area was examined by region (top, middle, or bottom), the appearance of cellular material and blood vessels was less evident in the ECM-ONLY than in the ECM-CELL. Values for the number of myofibers and blood vessels in the middle region were significantly lower than the values for the respective top and bottom regions that bordered the transected myofibers, although ECM-CELL middle region values were generally higher than the corresponding ECM-ONLY values (Figs. 5 and 7).

Discussion

The repair of a physical deformity after traumatic injury is important for the psychological well-being of victims.⁴¹ Therefore, developing an implant capable of filling in an area of missing tissue to prevent physical deformity is important, but the development of an implant capable of fully restoring function of the muscle in addition to cosmetic restoration of the area is the goal of tissue regeneration. To this end, this represents the first report of improved muscle function after repair of VML.

The data presented here prove that injuries involving VML that do not functionally regenerate without treatment can be surgically repaired with an MSC-seeded ECM. The treatment partially restores function, and the overall cosmetic appearance is similar to noninjured muscle. Whether longer periods of regeneration would have fully restored muscle fibers and blood vessels is not known; however,

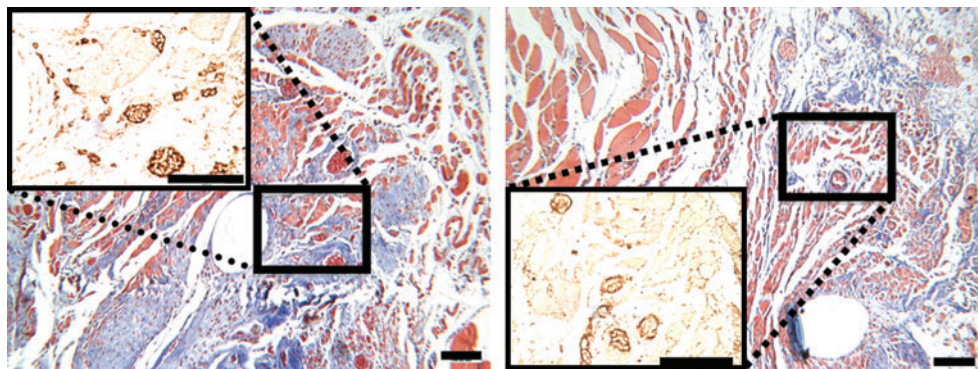


FIG. 4. von Willebrand factor (vWF). Masson's Trichrome stain of sections of implant from 42-day recovery of ECM-ONLY (left) and ECM-CELL (right) with insets demonstrating the appearance of vWF-positive blood vessels. Magnification: Trichrome = $200\times$; inset vWF = $400\times$. Scale bars = $100\mu\text{m}$. Color images available online at www.liebertonline.com/ten.

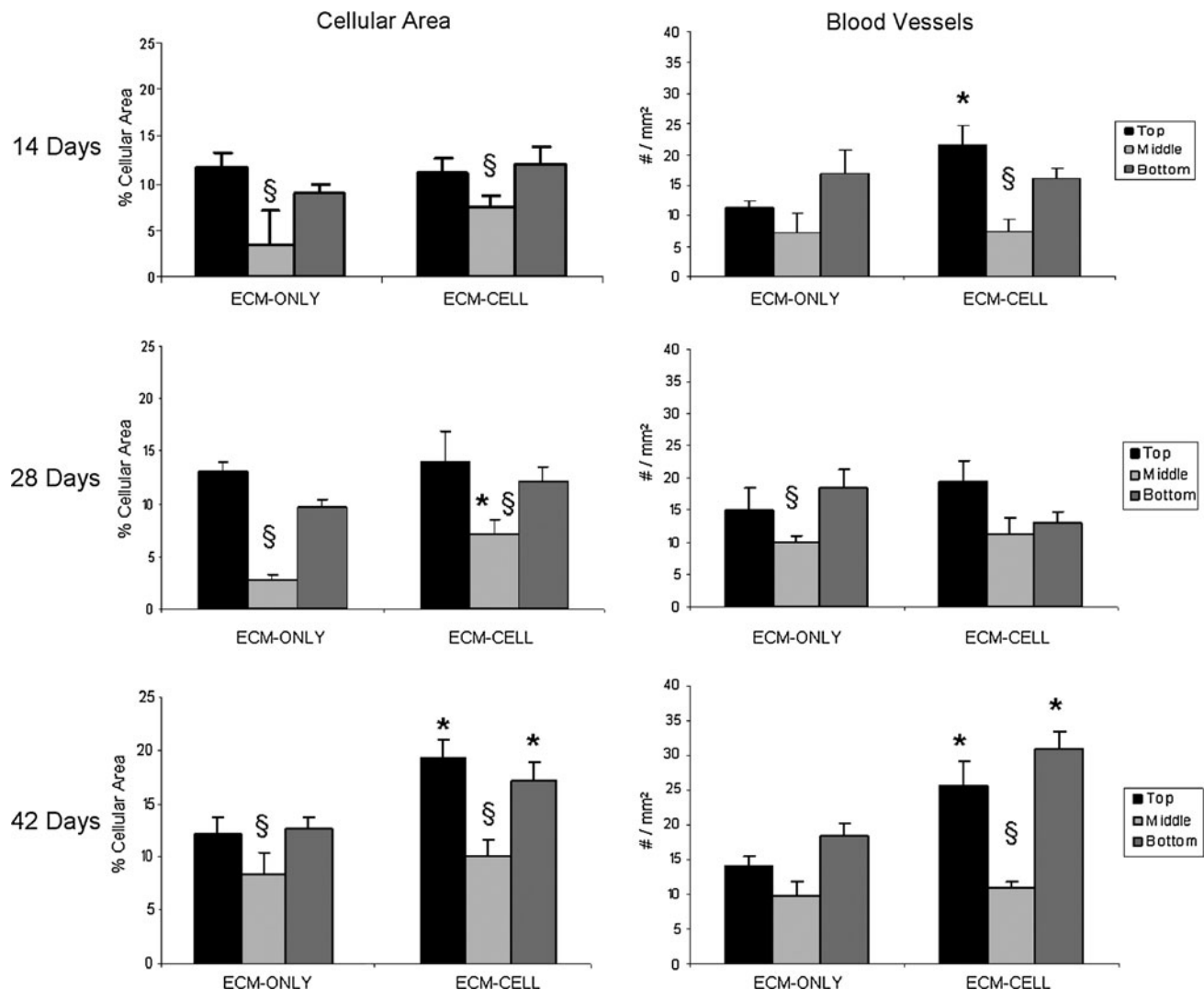


FIG. 5. Cellular area and blood vessels within defect. *Significantly different from same ECM-ONLY region ($p < 0.05$). §Significantly different from top and bottom ($p < 0.05$).

because there were so many myogenin-positive cells at 42 days, it is possible that the regeneration process was not complete, and given more time, these cells might have further contributed to functional recovery. Despite this, the fact that the muscle tissue that was restored had near-normal function per unit of cross-sectional area is critical for muscle regeneration. The functional restoration over 42 days when implanted ECMs were seeded with MSCs is associated with an increase in the number of blood vessels and myofibers growing within the implant, suggesting that the injected, homologous, bone-marrow-derived cells participate in the regeneration process. Whether the cells actually engraft or merely create an environment that enhances regeneration cannot be determined by these data.

The adherent fraction of cells derived from the bone marrow are generally considered to be a population of cells known as marrow stromal cells or MSCs that are multipotent and capable of differentiating into a number of different tissues.^{42,43} To confirm that the adherent bone muscle cells used in this study were MSCs, FACS analysis was performed. The cells were CD90⁺, CD45⁻, CD34⁻, and CD146⁻,

which is consistent with published reports of rat MSCs.⁴⁴⁻⁴⁶ Due to the multipotent nature of MSCs, they are a good cell population to use to aid in the regeneration of a loss of a large volume of tissue such as the muscle defect model used in this study. Another reason MSCs are an attractive cell therapy candidate is that they are easily obtained from the bone marrow and can be expanded in culture to provide clinically relevant quantities of cells. In fact, they are already in use in tissue regeneration applications clinically,⁴⁷ and point of care devices designed to harvest autologous bone marrow to provide isolated MSCs have been approved for clinical use in Europe and many Asian countries, and are currently seeking FDA approval in the United States.

The improved functional and histological regeneration observed after 42 days in the ECM-CELL group is likely the result of a number of different positive effects attributed to the implanted MSCs. Research from the lab of Palermo *et al.* proved that endogenous cells from the bone marrow participate in muscle regeneration due to physiologic stress.³⁴ While the participation of these bone marrow cells in muscle regeneration appears to be rare (<3.5%), they progress from

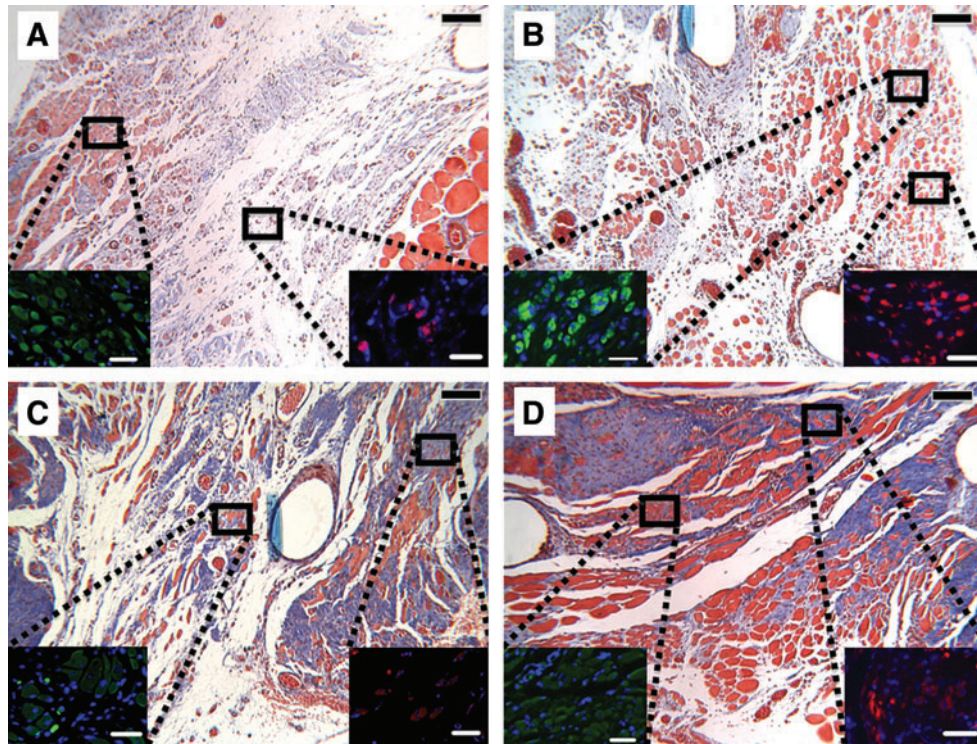


FIG. 6. Desmin and myogenin immunofluorescence. (A) ECM-ONLY at 28 days, (B) ECM-CELL at 28 days, (C) ECM-ONLY at 42 days, and (D) ECM-CELL at 42 days. Increased myofiber filtration, and desmin- and myogenin-positive structures with increasing time of recovery and after cell injection into the defect area. Stained with Masson's trichrome (center), desmin (lower left), and myogenin (lower right). Circular spaces are suture holes. Trichrome scale bar = 100 μ m, Desmin/myogenin scale bar = 50 μ m. Color images available online at www.liebertonline.com/ten.

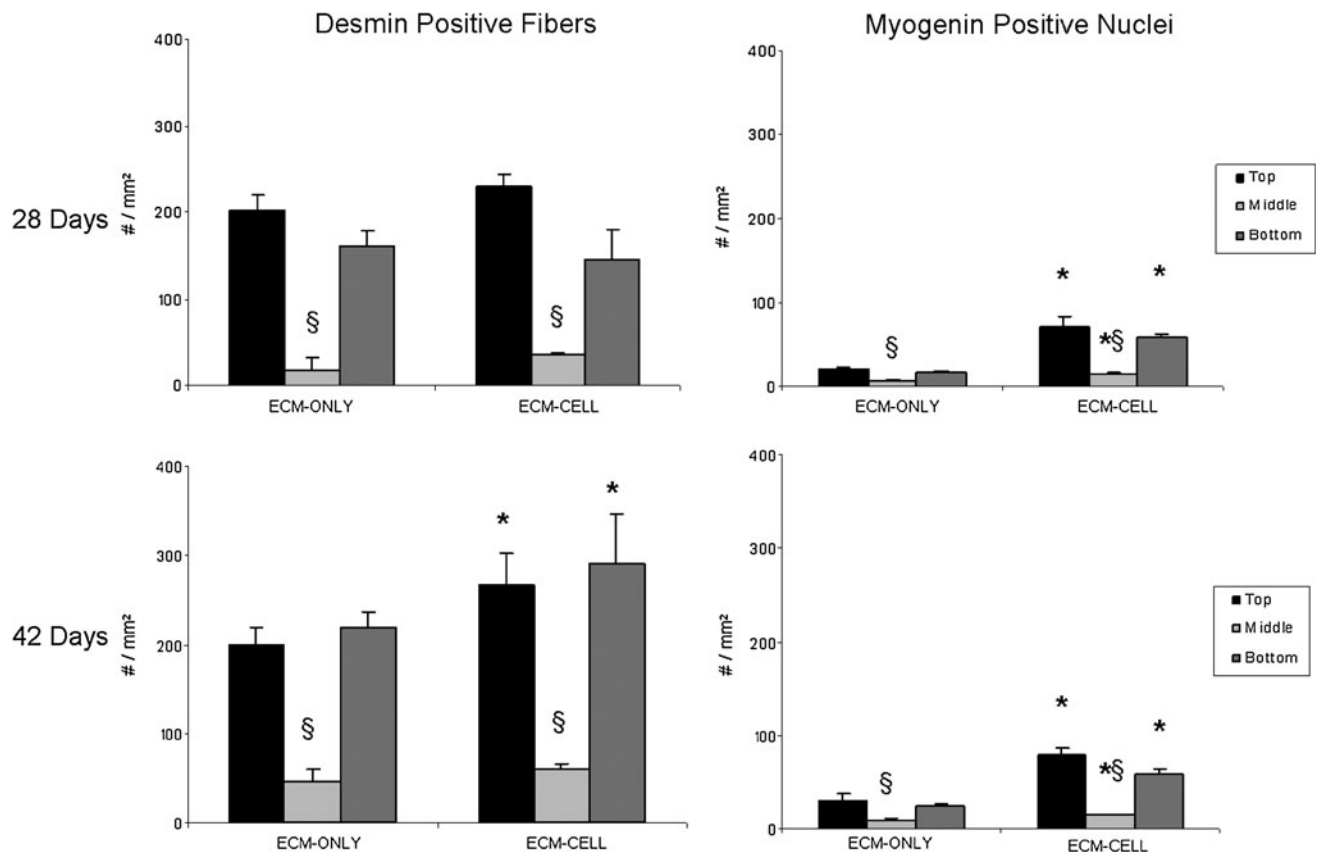


FIG. 7. Desmin-positive fibers and myogenin-positive nuclei. *Significantly different from same ECM-ONLY region ($p < 0.05$). §Significantly different from top and bottom ($p < 0.05$).

the bone marrow and into the muscle where they become muscle progenitor and/or satellite cells that can be activated in response to muscle injury.^{33,48} The addition of exogenous MSCs to dystrophic skeletal muscle is able to partially restore expression of dystrophin within the fibers.^{30,35,49,50} Conflict exists as to whether or not the addition of MSCs contributes to skeletal muscle as a result of differentiation into myofibers, fusion of MSCs with existing myofibers with or without differentiation, or by the secretion of trophic substances by the MSCs. Differentiation of MSCs along a myogenic lineage and fusion to form myotubes does occur *in vitro*,^{29,30,51,52} and there is also evidence that it occurs *in vivo*.^{49,53} Injury to skeletal muscle after irradiation and green fluorescent protein (GFP⁺) marrow replacement showed that as many as 12% of myofibers express GFP, indicating significant fusion, but these results might not translate to the direct injection of MSCs into injured areas.⁵⁴ Fusion events do appear to occur when tagged MSCs are injected directly into injured skeletal muscle, although they are relatively rare and it is difficult to determine whether or not the MSCs differentiated along a skeletal muscle lineage before fusion.⁵⁵ Some researchers have noted improvements in cardiac and skeletal muscle regeneration after stem cell treatment without either differentiation of the cells to a myogenic lineage or fusion with resident cells.^{37,56} MSCs release cytokines and growth factors such as vascular endothelial growth factor and improve vascularization and perfusion of damaged tissues, including skeletal muscle.⁵⁷ The increase in the number of blood vessels in the ECM-CELL after 42 days is likely a result of this mechanism. Further evidence for the paracrine actions of MSCs is the improved regeneration of cardiac muscle after infarction by the injection of a cell-free MSC conditioned medium into the infarct site.⁵⁸ Interestingly, recent evidence proves that MSCs need not even be in the local area of injury to exert their effects. Shabbir *et al.* injected MSCs or MSC-conditioned media into distant skeletal muscle and saw significantly improved function in a heart failure model.^{59,60} Even without actually tracking the cells injected in this study, it is likely that the beneficial effects are explained by the trophic factors released by the MSCs. Natsu *et al.* treated skeletal muscle laceration with bone marrow-derived MSCs, and, as was seen in this study, the muscle improved functionally without evidence of fusion or differentiation of the injected cells.³⁷

While this is the first time that MSCs have been seeded on a decellularized ECM for skeletal muscle regeneration, other myogenic progenitor cells have been seeded on decellularized ECMs implanted into skeletal muscle, although functional assessment of these has been limited. Similar to what others have seen with cell-seeded constructs of ECM implanted into defected skeletal muscle of the abdominal wall, the ECMs of the ECM-ONLY and ECM-CELL groups were both capable of supporting the growth of myofibers as well as blood vessels.^{23–25,27} Gamba *et al.*, however, did not have myofiber ingrowth into decellularized ECM constructs without addition of exogenous cells⁶¹ as opposed to the results in the ECM-ONLY groups. The VML model used here likely provides a more suitable environment for regeneration. The LGAS used in this model is an active, load-bearing muscle that is subjected to work during normal cage activity, whereas the rabbit's abdominal muscle defect in the Gamba *et al.* study is not subjected to the same relative functional

demands. Mechanical stimulation and stretch of damaged/regenerating myofibers is known to improve regeneration,⁶² and it is likely that the activity levels of the LGAS aided in the regeneration of myofibers into the defect area as was observed.

Terada *et al.* lacerated myofibers and fixed the distance between the transected ends to determine the maximal distance that they could grow to bridge the gap.⁹ A distance >2.3 mm was too far for the myofibers to bridge, which is consistent with what was seen in the ECM-ONLY LGAS. Despite the fact that cells were injected throughout the top, middle, and bottom of the ECM in the ECM-CELL group, only a limited number of desmin- and myogenin-positive fibers were found in this region. Both groups had did have myofiber ingrowth, but most blood vessels and myofibers were located in the top and bottom regions of the ECM implant area, <3 mm from the border of the ECM with the LGAS. Many of the cells expressed myogenin, indicating that they were newly regenerated myofibers. These myofibers were likely from the growth of injured myofibers into the ECM from the superior and inferior portions of muscle remaining after the defect injury. Since the injected MSCs were not tagged or tracked, differentiation and/or fusion of the MSCs cannot be ruled out, but the significantly higher number of fibers expressed in regions closer to the border with native muscle tissue indicate that engraftment of cells was not the main method of regeneration. Cells injected directly into the middle of the ECM would have been further away from a blood supply, and many of the cells could have died from lack of nutrients before the vascular supply grew to the area.

As an implanted ECM remodels, it releases factors that attract myogenic progenitor cells and stimulates their proliferation and differentiation.^{63–65} This, in combination with the trophic factors released by the bone marrow-derived cells, could stimulate the regeneration of myofibers transected during the creation of the defect, and this could explain the higher population of myofibers and blood vessels in the top and bottom region of the ECM relative to the middle region.

After 42 days, the regeneration of tissue into the injured area in the ECM-CELL group is capable of contributing to significant improvements in nerve-stimulated muscle function. This improvement of function implies that at least some of the regenerated myofibers were reinnervated, although nerve innervation was not specifically measured. Evidence exists that despite improvements in the short term, cell-seeded ECMs implanted into muscle might not show the same improvements over the long term.²⁴ Regenerating myofibers that are not reinnervated will degenerate.⁶⁶ Since the innervation state of the regenerating myofibers was not studied, the myofibers observed in the ECM might not be permanent and allowing longer recovery periods after the procedure might have yielded different results. Evidence in culture suggests that vascular endothelial growth factor, which is also released by MSCs, is capable of stimulating neurogenesis.⁶⁷ Future research, however, should focus on the neurotization of the constructs at the time of implantation as this is likely to improve function.⁶⁸

Another technique that could improve the function in this model further is the use of a larger number of cells. Winkler *et al.* determined that the addition of 1×10^7 MSCs to a severe

muscle crush injury significantly improved the functional recovery of the muscle more than 2.5×10^6 MSCs improved functional recovery.⁵⁵ Addition of only 1×10^6 MSCs did not show any significant functional improvement. In the present study, only 1.5×10^6 cells were injected into the $1 \times 1 \text{ cm}^2$ ECM repaired defect and significant functional improvement was observed. It is possible that additional functional improvement might have been stimulated by greater supplementation of MSCs, but a dose response was not carried out in this study.

Other labs in muscle engineering have focused primarily on bioreactor-based approaches in which three-dimensional muscle constructs are developed *in vitro* for future *in vivo* implantation.⁶⁹ However, to date, there are no reports of actually employing this approach to repair injured muscle. We have taken a different approach by first implanting ECM into the VML and then introducing MSCs, in effect, using the animal as a bioreactor. This approach offers significant logistical advantages over the bioreactor-based approach and is based on studies that have used stem cell-based therapy to repair injured⁷⁰ or diseased muscle.⁷¹

Additional regenerative or rehabilitative modalities utilized to enhance improvements in functional recovery using this model might include supplementation of growth factors to the defect area or by the use of physical therapy regimens involving mobilization and exercise to stimulate the regenerative process.

In conclusion, the data presented demonstrate for the first time the return of function to a large VML by the addition of MSCs seeded on a decellularized ECM implant. Translation of this technique to the clinic could significantly improve the lives of wounded military personnel and other patients who have lost large portions of muscle tissue.

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